Biosorption and bioreduction of Cr(VI) by locally isolated Cr-resistant bacteria

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Abstract Cr(VI) biosorption and bioreduction ability of locally isolated Cr-resistant bacteria was investigated using the shake-flask technique. A mixture of *S. epidermidis* and *B. cereus* showed the highest minimum inhibitory concentration (MIC) level at 750 mg/L Cr(VI) followed by *S. aureus* and *Bacillus sp.* of 250 mg/L, and *A. haemolyticus* of 70 mg/L. From the Langmuir adsorption isotherm, the treatment of cells with heat-acid resulted in the highest amount of Cr(VI) adsorped (78.25 mg/g dry wt. for *S. epidermidis*) compared to heat-acetone (67.93 mg/g dry wt. *Bacillus sp.*), heat only (36.05 mg/g dry wt. *S. epidermidis*) or untreated cells (45.40 mg/g dry wt. *S. epidermidis* and *B. cereus*). FTIR analysis showed the involvement of amine groups in Cr(VI) adsorption. In the bioreduction study, *A. haemolyticus* was able to completely reduce Cr(VI) up to 50 mg/L.

Keywords Bacteria; bioreduction; biosorption; chromium; FTIR

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Introduction

A wide array of technologies is available for the removal of chromium from wastewaters. Treatment of Cr (VI) waste usually consists of a two-stage process, i.e. chemical reduction of Cr (VI) to Cr (III) using sulphur dioxide, sodium bisulphite or sodium meta-bisulphite followed by precipitation of Cr (III) using either lime, caustic soda or sodium bicarbonate (Cushnie, 1985). Even though the process is efficient, large volumes of sludge are generated and the release of dangerous gases and expensive cost of the chemical reducing agents makes it imperative to look into safer and cheaper alternatives where a biological system seems to be the suitable approach. Numerous reports have demonstrated the feasibility of using biological processes for the treatment of Cr (VI) industrial effluents by either pure culture or a consortium of Cr (VI) reducing bacteria (Romanenko *et al.*, 1976; Bopp and Ehrlich, 1988; Lupton *et al.*, 1991, 1992; Turick and Apel, 1997). This paper discusses the Cr(VI) biosorption and bioreduction ability of five locally isolated Cr-resistant bacteria. Bacterial Cr(VI)-resistant ability was assessed using MIC. The Cr(VI) biosorption capacity of bacteria was evaluated using a Langmuir adsorption isotherm. FTIR analysis was carried out to identify functional groups present in bacteria responsible for Cr(VI) binding. The extent of Cr(VI) bioreduction by bacteria was determined via the diphenylcarbazide method.

Methods

Bacteria

In this work, five bacterial strains, which were isolated from metal-containing industrial effluents from various locations in Johor and Kota Bharu, Malaysia, were used (Table 1). All sampling, sample handling, cultivation, screening, characterisation and identification of the bacteria were carried out according to methods outlined in the *Standard Methods for the Examination of Water and Wastewater* (Greenberg *et al.*, 1992).

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Table 1 Profile for bacteria used in this study

Bacteria	Source	Gram	Identification method
Acinetobacter haemolyticus (A. haemolyticus)	Batek effluent, Kota Bahru	Negative	16S rRNA
Staphylococcus aureus(S. aureus)	Hospital discharge, Johor	Positive	API 20E identification kit
Bacillus cereus (B. cereus)	Hospital discharge, Johor	Positive	API 20NE identification kit
Staphylococcus epidermidis (S. epidermidis)	Hospital discharge, Johor	Positive	API 20E identification kit
Bacillus sp.	Textile effluent, Johor	Positive	Microlog® Biolog Identification System

Preparation of bacterial cell suspension and cell pre-treatment

Bacterial cell suspensions were prepared by harvesting cells after 24 h of growth. The cells were harvested by centrifugation (SIGMA 2K-15, B. Braun) at 9,000 rpm, 5 min and 0 °C. The pellets obtained were then washed twice using 0.85 g NaCl/100 mL solution at 9,000 rpm, 3 min and 0 °C and these were then resuspended in the same solution at 17.5% of the original volume of cell. Cell suspension (5 mL) was filtered through a hydrophobic-type 0.45 μm Whatman filter paper and dry wt. Was determined after overnight drying at 70–80 °C. For biosorption studies, cell suspensions were subjected to various treatments, namely heat via autoclaving at 121 °C for 15 min (*A. haemolyticus, S. epidermidis, Bacillus sp.*), heat-0.1%v/v acetone (*S. epidermidis, Bacillus sp.)* and heat-0.1N HCl *(S. epidermidis, Bacillus sp.). S. aureus* and *B. cereus* were not treated. For the bioreduction study, untreated cells of *A. haemolyticus* were used.

Determination of Cr(VI)-resistant ability of bacteria

Fresh NB medium (1.00–3.75 mL) was pipetted into a repli-plate dish (Sterilin, UK) followed by 0.25–4.75 mL of stock Cr (VI) solution (1,000 mg/ L) to give Cr (VI) concentrations ranging between 50 and 1,000 mg/ L. Stock Cr (VI) solution was prepared by dissolving 2.829 g $K_2Cr_2O_7$ (294.18 $gmol^{-1}$) in 1 L of deionised water (Elgastat, UHQII). The pH of Cr (VI) solution was adjusted to 7.0 using 0.1 M NaOH or 0.1 M HCl before filter-sterilising using 0.45 μm Whatman filter paper. A 5% v/v inoculum of the 24 h grown cell (in NB medium) was then added to the repli-plate and incubated at either 30 °C (*A. haemolyticus*, *A. calcoaceticus*, *Bacillus sp.* and *B. cereus*) or 37 °C (*S. epidermidis*, *S. aureus*) for 7 days (Memmert, USA). The control experiment consisted of bacterial cells in NB medium, NB medium only and Cr (VI) in NB medium. The first concentration of Cr (VI) that totally inhibited bacterial growth was taken as the minimum inhibitory concentration (MIC). The MIC or resistant level of bacteria to Cr (VI) was evaluated by measuring percentages of cell survival at OD_{600} (Spectronic 21D) after 7 days of incubation. The experiment was performed in duplicate.

Biosorption of Cr(VI)

A series of Cr(VI) solutions ranging from 10–500 mg/L were prepared from Cr(VI) stock solution (1,000 mg/L) in 25 mL volumetric flasks. The volume was made up to 25 mL using deionised water prior to pH adjustment to 2.0 using 0.1 M HCl. One particular set of Cr(VI) solution was adjusted to pH 7.0. The experiment was initiated by adding, 20 mg cell dry wt. of either treated or untreated cell suspensions to the respective flasks before incubating at room temperature at 100–200 rpm between 2–24 hours. All experiments were performed in duplicate. The Cr(VI) adsorption was determined using an atomic absorption spectrometer (AAS, Philips PU8300) and was evaluated using the

Langmuir adsorption isotherm as described in Equation 1.

$$q_e = Q_o bc/1 + bc \quad (1)$$

where q_e is the Cr(VI) adsorped at equilibrium condition (mg/g cell dry wt.), Q_o is the maximum Cr(VI) adsorped (theoretical), b is the constant for adsorption energy coefficient and c is the Cr(VI) concentration at equilibrium.

FTIR analysis was carried out to investigate the role of functional groups present on bacterial cell walls in Cr(VI) adsorption. For this reason, cells of *B. cereus* before and after contact with 200 mg/L Cr(VI) was analysed. A dried cell pellet (1 mg) was mixed with 200 mg KBr (Scharlau). The mixture was vacuum-pressed and the disk recovered was immediately analysed with an FTIR spectrophotometer (FTIR, 8300, Shimadzu) between 4000 and 400 cm^{-1}.

Bioreduction of Cr(VI)

The cell suspension of *A. haemolyticus* (10 mg cell dry wt.) was mixed with 100 mL NB medium in 250 mL Erlenmeyer flasks and grown for 12 h. Then, Cr(VI) at 10–100 mg/ L was added to the mixture where Cr(VI) reduction was evaluated at 0, 0.25, 0.50, 1, 2, 4, 8, 12, 24, 36 and 48 h. The hyperbolic equation of Michaelis–Menten, $V = V_{max}{}^{*}[S]/K_m + [S]$, where [S] represents Cr(VI) concentration, V_{max} is maximum reaction velocity and K_m is the Michaelis constant, was used to determine the kinetic parameters (K_m and V_{max}) of the reaction. Evaluation was based on the assumption of a pseudo first-order rate reaction. Mixtures of NB medium and Cr(VI) minus the bacterial cell acted as control. The reduction of hexavalent chromium was determined colorimetrically at 540 nm using the diphenylcarbazide (DPC) method with a detection limit of 5 μg L^{-1}. In a 10 mL volumetric flask, 1 mL of sample was mixed with 9 mL of 0.2 M H_2SO_4, then 0.2 mL of freshly prepared 0.25% (w/ v) DPC in acetone was added to the volumetric flask. The mixture was then vortexed (Maxi Mix-II Thermolyne) for approximately 15–30 s and left to stand for between 10 and 15 min for full colour development. The red-violet to purple colour formed was then measured at OD_{540} using distilled water as reference. The instrument used was calibrated using 0.4–2.0 mg/ L Cr(VI) prepared from Cr(VI) stock solution (1,000 mg/ L).

Results and discussion

Determination of Cr(VI)-resistant ability of bacteria

A mixture of *S. epidermidis* and *B. cereus* showed a higher resistant level to Cr(VI) with MIC condition only achieved at 750 mg/L Cr(VI). This was followed by *S. aureus* and *Bacillus sp.* with MIC of 250 mg/L and *A. haemolyticus* (70 mg/L). This result shows that Gram positive bacteria generally have higher Cr(VI) resistant ability and mixed cultures are more resistant to Cr(VI) compared to single culture. The former observation is attributable to the thicker cell wall of the Gram positive strains, which is made up of 90% peptidoglycan units. (Madigan *et al.*, 2001). As a comparison, only approximately 10 percent of the cell wall of Gram negative strain is made up of peptidoglycan. The amino acids present in peptidoglycan have the ability to detoxify Cr(VI) via binding, hence preventing it being reduced to Cr(III) inside the cells. This is because, during the step-wise three electron reduction of Cr(VI) to Cr(III), reactive oxygen species (ROS) such as $\cdot O_2^-$, H_2O_2 and $\cdot OH$ will be formed where these ROS may directly trigger DNA alterations that include DNA strand breaks and dG hydroxylation (Ye *et al.*, 1999). Therefore, the Gram positive strains with the thicker cell wall and higher content of the amino acids would literally have higher Cr(VI) resistant ability. Higher Cr(VI) resistance by mixed cultures compared to single strain can be attributed to various types of interactions between the bacterium that may occur such as neutralism, co-mensalism and mutualism (Madigan *et al.*,

Table 2 Langmuir adsorption parameters for biosorption of Cr(VI) by bacteria

Bacteria	Treatment	pH	Initial Cr(VI), mg/L	*Q*o (theoretical), mg/g dry wt.	q_e (experimental), mg/g dry wt.	*b*	R^2
A.haemolyticus	Heat	2.0	10–100	38.91	12.34	0.0048	0.9821
S.aureus + *B.cereus*	Untreated	7.0	20–400	714.29	45.40	0.00039	0.9727
S.epidermidis	Heat			47.17	36.05	0.0081	0.7681
	Heat-acetone	2.0	20–500	113.64	54.84	0.0025	0.9736
	Heat-acid			128.21	78.25	0.0034	0.9739
B.cereus	Heat			37.31	30.23	0.0100	0.6661
	Heat-acetone	2.0	20–500	101.01	67.93	0.0046	0.8862
	Heat-acid			138.89	77.62	0.0032	0.9774

2001). These interactions generate metabolic by-products that can either reduce Cr(VI) to Cr(III) or bind with Cr(VI), rendering it inactive to enter cells (Nies *et al.*, 1998).

Biosorption of Cr(VI)

A combination of heat-acid treatment resulted in the highest amount of Cr(VI) adsorped compared to heat-acetone, heat only or untreated cells (Table 2). This was due to acid hydrolysis that results in protonation of the weakly basic amine group of amino acids that constitute a large portion of the bacterial cell wall structure, thus exposing more binding sites for the accessibility of Cr(VI) in the form of oxyanions (Sudha Bai and Abraham, 2002).

Heat-acetone treated cells also showed high Cr(VI) sorption capacity that can be attributed to increased surface area from the removal of protein and lipid fractions from the cell wall structure. A similar finding was also reported by Sudha Bai and Abraham (2002). Low Cr(VI) binding by the untreated cells does not necessarily indicate its ineffectiveness as a biosorbent as parameters such as time and Cr(VI) concentrations were not optimum. An immense difference between the q_e and *Q*o values indicate that the adsorption may proceed at higher Cr(VI) concentrations or requires a longer time before equilibrium. The adsorption energy coefficient (*b*) obtained from the Langmuir adsorption isotherm can be used to predict the potential of bacteria as biosorbent for Cr(VI). Highly potential biosorbents are characterised by high q_e value and low b, which usually accompanies the favourable steep initial isotherm slope (Volesky, 1990). An example of the use of Langmuir adsorption isotherm in Cr(VI) binding by *B cereus* is shown in Figure 1.

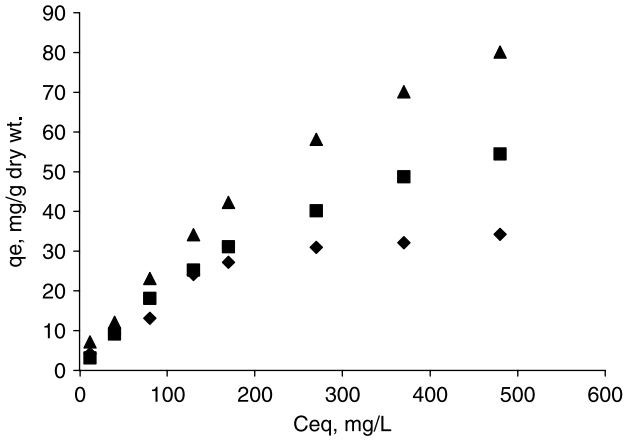

Figure 1 Biosorption of Cr(VI) by *B. cereus*; ▲, heat-acid, ■, heat-acetone, ◆, acid; Cr(VI), 20–500 mg/L, pH 2.0, shaken at 100 rpm for 7 h

The involvement of a weakly basic amine group of amino acids from bacteria can also be supported by FTIR analysis. Example of FTIR spectrum depicting this condition is shown in Figures 2 and 3.

There was a notable shift for the amine group upon contact with Cr(VI) i.e. 3,433 to 3,131 cm^{-1} as opposed to the C═O based groups (1,657 to 1,654 cm^{-1}). This can be explained as follows: Cr(VI) exists as negatively charged oxoanions in aqueous environment. Since the experiment was conducted at pH 2.0, complete protonation of the amine groups (NH_2 to NH_3^+) would occur allowing an electrostatic interaction with the negatively charged Cr(VI) ions. The binding of chromium with a considerably high atomic number would literally reduce the vibration of the RNH-Cr complex, thus shifting the stretching vibration to a lower position (Sudha Bai and Abraham, 2002; Volesky and Kuyucak, 1989). At the same time, the carboxyl groups would be fully dissociated to COO^-, hence eliminating its role in binding of the negatively charged Cr(VI) ions.

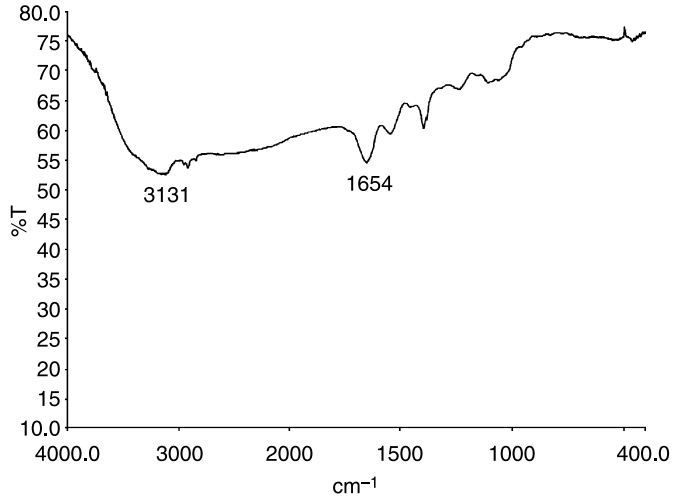

Figure 2 FTIR spectrum of heat-treated cells of *B. cereus* in 200 mg/L Cr(VI)

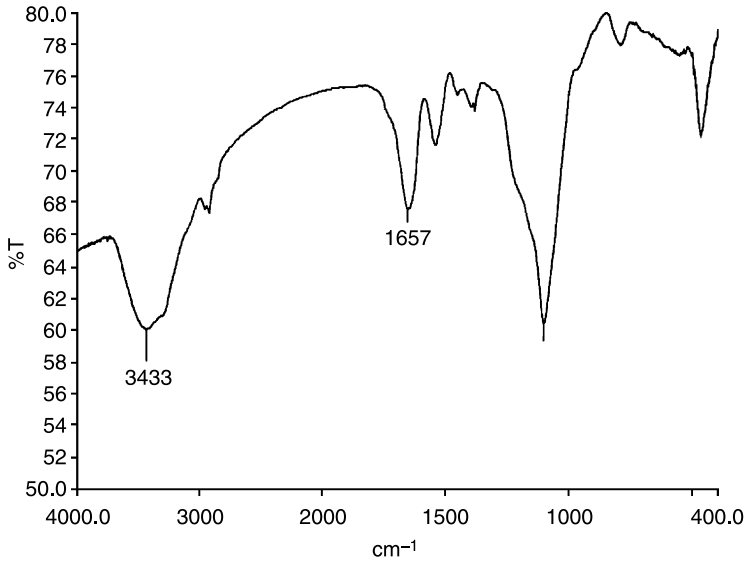

Figure 3 FTIR spectrum of heat-treated cells of *B. cereus* without Cr(VI)

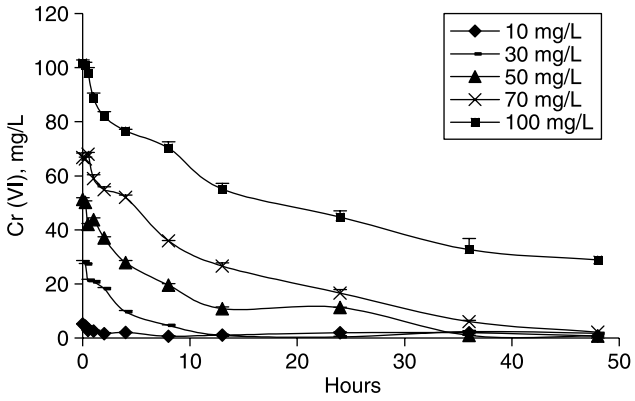

Figure 4 Bioreduction of Cr(VI) by *A. haemolyticus*

Bioreduction of Cr(VI)

A. haemolyticus was able to completely reduce Cr(VI) up to initial Cr(VI) concentrations of 50 mg/ L (Figure 4). Around 8, 13 and 36 h was needed to completely reduce 10, 30 and 50 mg/L Cr(VI), respectively. An apparent Michaelis–Menten constant, K_m of 77.52 mg/L CrO_4^{2-}(18.29 mM) and a maximum velocity, V_{max} of 2.26 μg CrO_4^{2-} L^{-1} min^{-1} mg^{-1} cell dry wt. were obtained from Lineweaver–Burke plots. The values were derived from the assumption that with excess carbon source in NB medium, the reaction would be pseudo first order and the initial reaction rate would be independent of the substrate i.e. Cr(VI) concentration (McLean and Beveridge, 2001).

The reduction rate decreased gradually at Cr(VI) concentrations higher than 50 mg/L. At an initial Cr(VI) concentration of 70 mg/L, 88% of Cr(VI) was reduced while the value further decreased to 75% at 100 mg/L Cr(VI) after 48 h reaction time. The rate of Cr(VI) reduction decreased with time and eventually ceased at higher Cr(VI) concentrations. The inability of *A. haemolyticus* to completely reduce 70 and 100 mg/L Cr(VI) indicates the existence of a finite Cr(VI) reduction capacity, which is a result from Cr(VI) toxicity towards biological activity (Wang and Xiao, 1995). The same situation was also reported by Philip *et al.* (1998) where even though Cr(VI) reduction by *Bacillus coagulans* occurred at the highest Cr(VI) concentrations, complete Cr(VI) reduction was not observed for an initial concentration higher than 26 mg/L over 72 h. A similar case was reported by Wang and Xiao (1995) where *Bacillus sp.* was unable to reduce Cr(VI) completely at Cr(VI) higher than 20 mg/L in 96 h. The ability of *A. haemolyticus* to reduce 10 mg/L Cr(VI) in an 8 h period is of interest as it is relatively fast compared to other species. For example, Laxman and More (2002) reported that 30 h was needed by *Streptomyces griseus* to completely reduce 10 mg/L Cr(VI), while Rege *et al.* (1997) reported that *Enterobacter cloacae* reduced 5 mg/L Cr(VI) after 24 h. One possible reason for *A. haemolyticus* having a faster Cr(VI) reduction ability than *Streptomyces griseus* is the better Cr(VI) resistance ability.

Conclusion

The Cr(VI) biosorption and bioreduction ability of locally isolated Cr-resistant bacteria, identified as *S. epidermidis*, *B. cereus*, *S. aureus*, *Bacillus sp.* and *A. haemolyticus*, was successfully demonstrated. Combination of heat-acid treatment gives the highest Cr(VI) removal compared to untreated cells. Cr(VI) adsorption was due to the amine groups present as shown by the FTIR analysis. Further understanding on the properties of these bacteria should greatly assist towards its application in chromium bioremediation processes.

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References

Bopp, L.H. and Ehrlich, H.L. (1988). Cr(VI) resistance and reduction in *Pseudomonas fluorescens* strain LB300. *Arch. Microb.*, **150**, 426–431.

Cushnie, G.C. Jr. (1985). *Electroplating Wastewater Pollution Control Technology*, Noyes Publications, New Jersey, USA.

Greenberg, A.E., Trussell, R.R. and Clesceri, L.S. (1992). *Standard Methods for the Examination of Water and Wastewater*, 18th ed., American Public Health Association, New York.

Laxman, R.S. and More, S. (2002). Reduction of hexavalent chromium by *Streptomyces griseus*. *Min. Eng.*, **15**, 831–837.

Lupton, F.S., DeFilippi, L.J. and Goodman, J.R. (1991). Bioremediation of chromium (VI) contaminated aqueous systems by sulfate reducing bacteria. US Patent 5062956.

Lupton, F.S., DeFilippi, L.J. and Goodman, J.R. (1992). Bioremediation of chromium (VI) contaminated solid residues. US Patent 5155042.

Madigan, M.T., Martinko, J.M. and Parker, J. (2001). *Brock: Biology of Microorganisms*, 9th ed., Prentice-Hall International, New York.

McLean, J. and Beveridge, T.J. (2001). Cr(VI) reduction by a *Pseudomonad* isolated from a site contaminated with chromated copper arsenate. *Appl. Environ. Microb.*, **67**(3), 1076–1084.

Nies, D.H., Koch, S., Wachi, S., Peitzsch, N. and Saier, M.H. Jr. (1998). CHR, a novel family of prokayotic proton motive force-driven transporters probably containing chromate/sulfate antiporters. *J. Bac.*, **180**, 5799–5802.

Philip, L., Iyengar, L. and Venkobachar, C. (1998). Cr(VI) reduction by *Bacillus coagulans* isolated from contaminated soils. *J. Env. Eng.*, **124**(12), 1165–1170.

Rege, M.A., Petersen, J.N., Johnstone, D.L., Turick, C.E., Tonge, D.R. and Apel, W.A. (1997). Bacterial reduction of hexavalent chromium by *Enterobacter cloacae* strain HO1 grown on sucrose. *Biotech. Lett.*, **19**, 691–694.

Romanenko, V.I., Kuznetsov, S.I. and Korenkov, V.I. (1976). Method of biological purification of industrial effluents from chromates and bichromates. US Patent 3941691.

Sudha Bai, R. and Abraham, E.T. (2002). Studies on enhancement of Cr(VI) biosorption by chemically modified biomass of *Rhizopus nigricans*. *Wat. Res.*, **36**, 1224–1236.

Turick, C.E. and Apel, W.W. (1997). Method for in situ or ex situ bioremediation of hexavalent chromium contaminated soils and/or groundwater. US Patent 5681739.

Volesky, B. (1990). *Biosorption of Heavy Metals*, CRC Press, Boca Raton. Florida.

Volesky, B. and Kuyucak, N. (1989). The elution of gold sequestered on a natural biosorbent. *Biorecov*, **1**, 205–218.

Wang, Y.T. and Xiao, C. (1995). Factors affecting hexavalent chromium reduction in pure cultures of bacteria. *Wat. Res.*, **29**(11), 2467–2474.

Ye, J., Wang, S., Leonard, S.S., Sun, Y., Butterworth, L., Antonini, J., Ding, M., Rojanasakul, Y., Vallyathan, V., Castranova, V. and Shi, X. (1999). Role of reactive oxygen species and p53 in chromium (VI)-induced apoptosis. *J. Biol. Chem.*, **274**(49), 34974–34980.